Inhibition of IL-12 production by 1,25-dihydroxyvitamin D3. Involvement of NF-kappaB downregulation in transcriptional repression of the p40 gene.

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Research Article

Interleukin 12 (IL-12), produced by myelomonocytic cells, plays a pivotal role in the development of T helper 1 (Th1) cells, which are involved in the pathogenesis of chronic inflammatory autoimmune disorders. 1,25-Dihydroxyvitamin D3 [1,25(OH)2D3] inhibits IL-12 production by activated macrophages and dendritic cells, thus providing a novel interpretation to its immunosuppressive properties. 1,25(OH)2D3 significantly inhibits mRNA expression for both IL-12 p35 and p40 subunits acting at the transcriptional level. The effect of 1,25(OH)2D3 on p40 promoter activation was analyzed by cotransfecting monocytic RAW264.7 cells with p40 promoter/reporter constructs and expression vectors for vitamin D3 receptor (VDR) and/or retinoid X receptor (RXRalpha). We observed transcriptional repression of the p40 gene by 1,25(OH)2D3, which required coexpression of VDR with RXR and an intact VDR DNA-binding domain. The repressive effect maps to a region in the p40 promoter containing a binding site for NF-kappaB (p40-kappaB). Deletion of the p40-kappaB site abrogates part of the inhibitory effect on the p40 promoter, confirming the functional relevance of this site. Activation of monocytic THP-1 cells in the presence of 1,25(OH)2D3 results in reduced binding to the p40-kappaB site. Thus, 1,25(OH)2D3 may negatively regulate IL-12 production by downregulation of NF-kappaB activation and binding to the p40-kappaB sequence.

Inhibition of IL-12 Production by 1,25-Dihydroxyvitamin D_3

Involvement of NF-κB Downregulation in Transcriptional Repression of the p40 Gene

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Abstract

Interleukin 12 (IL-12), produced by myelomonocytic cells, plays a pivotal role in the development of T helper 1 (Th1) cells, which are involved in the pathogenesis of chronic inflammatory autoimmune disorders. 1,25-Dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] inhibits IL-12 production by activated macrophages and dendritic cells, thus providing a novel interpretation to its immunosuppressive properties. 1,25$(OH)_2D_3$ significantly inhibits mRNA expression for both IL-12 p35 and p40 subunits acting at the transcriptional level. The effect of 1,25$(OH)_2D_3$ on p40 promoter activation was analyzed by cotransfecting monocytic RAW264.7 cells with p40 promoter/reporter constructs and expression vectors for vitamin D_3 receptor (VDR) and/or retinoid X receptor (RXRα). We observed transcriptional repression of the p40 gene by 1,25$(OH)_2D_3$, which required coexpression of VDR with RXR and an intact VDR DNA-binding domain. The repressive effect maps to a region in the p40 promoter containing a binding site for NF-κB (p40-κB). Deletion of the p40-κB site abrogates part of the inhibitory effect on the p40 promoter, confirming the functional relevance of this site. Activation of monocytic THP-1 cells in the presence of 1,25$(OH)_2D_3$ results in reduced binding to the p40-κB site. Thus, 1,25$(OH)_2D_3$ may negatively regulate IL-12 production by downregulation of NF-κB activation and binding to the p40-κB sequence. (*J. Clin. Invest.* 1998. 101:252–262.)

Key words: VDR • NF-κB • T helper • antiinflammatory drug • autoimmunity

Introduction

IL-12 production, by activated myelomonocytic cells, is critical for the development of T helper 1 (Th1)[1] cells and the initiation of cell-mediated immune responses to pathogens (1–4). This cytokine is a powerful inducer of IFN-γ secretion by T and natural killer (NK) cells, it acts as a growth factor for both cell types and enhances their cytolytic activity (5, 6).

IL-12 is a heterodimeric cytokine composed of two disulfide linked subunits of 35 (p35) and 40 (p40) kD encoded by two separate genes (7–9). Inducible expression of IL-12 has been documented in macrophages and dendritic cells (DC) after stimulation by microbial antigens or via CD40–CD40L interaction (10, 11). The expression of the p40 subunit is highly inducible and is regulated primarily at the transcriptional level (12, 13). A region extending 292 bp 5′ from the transcription initiation site confers inducibility to the p40 gene (12). Two major regulatory sites have been identified which bind inducible proteins belonging to the ets (13) and NF-κB (14) families of transcription factors. Expression of p35 is also subject to similar regulation, although to a much lesser extent than p40 (12, 15, 16).

The unique ability of IL-12 to direct Th1 development and cellular immunity explains its key role in the development of certain inflammatory autoimmune disorders (17). Indeed, inhibiting the action of IL-12 has been shown to prevent development and block progression of disease in experimental models of autoimmunity (18–23). These findings have raised great interest in identifying inhibitors of IL-12 production (24), which could be used in the control of chronic inflammatory autoimmune disorders. Recently, 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), the biologically active metabolite of vitamin D_3, has been reported to inhibit IL-12 production by stimulated macrophages (25). 1,25$(OH)_2D_3$ is required for normal calcium and phosphorus homeostasis and regulation of bone remodeling (26). In addition, 1,25$(OH)_2D_3$ has multiple effects on the differentiation and function of hematopoietic cells (27). Besides promoting the differentiation of promyelocytes into monocytes (28–31), the hormone inhibits T cell activation both in vitro and in vivo, and inhibits the secretion of IL-2, IL-1, IL-6, TNF, and IFN-γ (32–34).

1,25$(OH)_2D_3$ is a secosteroid hormone that binds to a nuclear receptor named vitamin D_3 receptor (VDR) (35, 36). Once bound to the hormone the receptor associates with specific recognition sequences, called vitamin D responsive elements (VDRE), which are present in the promoter region of target genes and are involved in regulating their transcription (37). Besides binding to certain VDREs as VDR homodimers (38, 39), this receptor can heterodimerize with the nuclear receptor for 9-*cis* retinoic acid, namely retinoid X receptor (RXR) (40, 41), to modify its DNA binding affinity to and the transcriptional response from particular VDREs (39, 41, 42).

Recent studies have shed some light into the molecular basis of the immunomodulatory activity of 1,25$(OH)_2D_3$. It has been reported that repression of IL-2 gene transcription by 1,25$(OH)_2D_3$ occurs via VDR-dependent inhibition of NFATp/AP-1 complex formation (43). In addition, it has also been described that 1,25$(OH)_2D_3$ mediates downregulation of

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1. *Abbreviations used in this paper:* DC, dendritic cells; NK, natural killer; RXRα, retinoid X receptor; SAC, *staphylococcus aureus* Cowan I; Th1, T helper 1; VDR, vitamin D_3 receptor; VDRE, vitamin D responsive element.

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NF-κB activation by decreasing NF-κB p50 and c-Rel protein expression in T cells (44). These findings suggested that $1,25(OH)_2D_3$ may inhibit IL-12 secretion by similar mechanisms.

Here we report that $1,25(OH)_2D_3$ inhibits IL-12 production by macrophages and DC by suppressing transcriptional activation of the p35 and p40 genes. We demonstrate that transcriptional repression of the p40 gene is dependent on expression of VDR with its heterodimeric partner RXR and requires functional DNA binding of VDR. Importantly, this inhibition is dependent, at least in part, on downregulation of NF-κB activation and binding to a κB sequence identified within the p40 promoter.

Methods

Cell lines and cell culture reagents. Human THP-1 and murine RAW 264.7 monocytic tumor cell lines (American Type Culture Collection, Rockville, MD) were cultured in RPMI 1640 medium supplemented with 5% FCS (Hyclone, Logan, UT), glutamine (2 mM), streptomycin (10 μg/ml), penicillin (10 U/ml), and sodium pyruvate (1 mM) (complete medium). J558L CD40L expressing cells (45) (gift of Dr. Peter Lane, Basel Institute for Immunology, Basel, Switzerland) were grown in Iscove's Modified Dulbecco's medium complete medium supplemented with L-histidinol dihydrochloride (5 mM) (Sigma Chemical Co., St. Louis, MO). The parental J558L cells were cultured in the same medium in the absence of L-histidinol.

Monocytes and dendritic cell preparation. Human monocytes were purified from buffy coats as previously described (46). Immature DC were prepared as described (47). Briefly, PBMC were isolated from a buffy coat by Ficoll (Pharmacia Biotec AB, Uppsala, Sweden), and incubated for 30 min with anti-CD19 antibodies (PharMingen, San Diego, CA), then washed. Goat anti–mouse IgG microbeads (Miltenyi Biotec GmbH, Bergish Gladbach, Germany) were added and after 30 min the cell suspension was applied on the top of a MiniMACS column (Miltenyi Biotec GmbH). B cell–depleted PBMC were plated in 25-cm^2 flasks at 6×10^6 cells/ml in complete medium for 2 h. Then nonadherent cells were removed with a gentle wash and complete medium supplemented with GM-CSF (800 U/ml) and IL-4 (1,000 U/ml) was added. Cells were analyzed on day 6 for CD1a and CD40 expression using a FACScan® (Becton-Dickinson, San Jose, CA). 7×10^4 DC were incubated with 3.5×10^4 irradiated (10,000 rad) J558L-mCD40L and IFN-γ (1,000 U/ml) in the presence or absence of $1,25(OH)_2D_3$. After 24 h, supernatants were collected and IL-12 and p40 concentrations were measured by ELISA.

Cytokine assays. ELISAs for IL-12 p40 and p75 were performed as described (48). Human recombinant IL-12, IL-12 p40, and anti–IL-12 mAbs were a gift from Dr. Maurice Gately (Hoffman-La Roche, Nutley, NJ). Human IL-10 was detected using ELISA kits (PharMingen) according to the manufacturer's instructions.

Plasmids. To construct the human p40 promoter/luciferase reporter constructs, we generated various p40 promoter fragments by PCR of genomic DNA obtained from THP-1 cells using TRIzol™ reagent (GIBCO BRL, Gaithersburg, MD). The resulting PCR products were ligated in pCR™ II (Invitrogen, San Diego, CA) and subsequently excised and religated in BamHI/XhoI sites of the PXP2 luciferase vector (49). To create the internal deletion of the p40-κB site (ΔκB), a PCR product extending from −106 to +56 bp was ligated in BamHI/XhoI sites of PXP2 after the procedure described above. Subsequently, a second PCR product extending from −111 to −292 bp and flanked by BamHI sites was ligated into the BamHI site of the −106/+56 p40-PXP2 vector. All inserts were verified by dideoxy DNA sequencing. Expression vectors for wild-type human VDR pCMV-hVDR and for VDR DNA-binding domain mutant pCMV-hVDR-R50G in which R-50 was mutated to G, were a gift of Dr. Leonard Freedman (Memorial Sloan-Kettering Cancer Center, New York). Vector for expression of human RXRα and empty vector PSG5 were described elsewhere (38). All plasmids were ampicillin resistant, grown in *Escherichia coli* and purified using a plasmid preparation kit (Qiagen Inc., Chatsworth, CA).

Oligonucleotides. The sequences of oligonucleotides used for PCR were: -292 5′-ataggatcCTGTCTCCAAGCACCTTGGC, -250 5′-ataggatccAGCATCTCCATCTCCTTCC, -224 5′-ataggatCCACCCAAAAGTCATTTCCTC, -180 5′-ataggatccTGTCTATGTTCCCTCCTCG, +56 3′-atactcgagTGCTCTGGGCAGGACGGAG, -104(κB-) 5′-ataggatCCCAGAAGGTTTTGAGAGTTG, -111(κB-) 3′-atacctaggTTTGAAGAAGTTCCTTTTTTGTTTGTC. The indicated base pair positions are relative to the p40 transcription initiation site, restriction sites are underlined, and p40 promoter sequences are capitalized. For the p40-κB used in EMSA, the sequence of the sense strand oligonucleotide was 5′-gatcCTTGAAATTCCCCCAG. The sequences of consensus and mutant NF-κB oligonucleotides were 5′-AGTTGAGGGGACTTTCCCAGGC (sense strand) and 5′-AGTTGAGGCGACTTTCCCAGGC (sense strand), respectively (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Oligonucleotide sequence of the octamer binding site from the human H2b was agctCTTCACCTTATTTGCATAAGC (sense strand). Oligonucleotides were purchased from Primm s.r.l. (Milan, Italy).

Northern blot hybridization and RNase protection assay. For Northern blots, total RNA was extracted from induced and untreated PBMC by TRIzol™ (Life Technologies Inc., Grand Island, NY). Equal amounts of RNA (10 μg/lane) were fractionated on a 1.5% agarose-formaldehyde gel. The specific mRNAs were detected by hybridization of Hybond N+ nylon membranes (Amersham Corp., Arlington Heights, IL) with ^{32}P-labeled cDNA probes for IL-12 p40, p35, and glyceraldehyde-3-phosphate dehydrogenase. The filters were exposed to X-Omat AR film (Eastman Kodak Co., Rochester, NY) between double intensifying screens (DuPont Co., San Diego, CA). For RNase protection assays, a 301-bp and a 265-bp PCR product derived, respectively, from the p40 and p35 cDNA were cloned into pCR™ II (Invitrogen). The vectors were linearized with appropriate restriction enzymes and transcribed with SP6 polymerase into complementary RNA (antisense) riboprobes using ^{32}P-CTP (Amersham Corp.) and the riboprobe kit (Promega Corp., Madison, WI). Cells were collected by low-speed centrifugation and disrupted at final concentration of 10^7 cells/ml of lysis/denaturation solution, according to the instructions of the Direct Protect™ lysate ribonuclease protection assay kit (Ambion Inc., Austin, TX). 45 μl of cell lysate was hybridized in solution with an excess of riboprobes (10^5 cpm for p40, 2×10^5 cpm for p35, 10^4 cpm for 18S) at 37°C overnight, and the instructions of Direct Protect™ kit were followed. The protected fragments were fractionated on 5% polyacrylamide/urea sequencing gel and detected by autoradiography.

Nuclear run-on assay. Isolation of nuclei and in vitro nuclear transcription were performed as described (50). In all experiments, equal cpm of ^{32}P-labeled RNA were hybridized to 1 μg of denatured plasmid DNA previously immobilized on N-Hybond transfer membrane (Amersham Corp.) by the use of a dot-blot apparatus.

Transfection by electroporation. Log phase RAW264.7 cells were harvested, washed twice, and resuspended in RPMI 1640 at a concentration of 2×10^7 cells/ml. 0.6 ml of cell suspension was then mixed with DNA, kept on ice for 5 min and electroporated in 0.45-cm electroporation cuvettes at 960 μF, 400 V using a Gene pulser apparatus (Bio-Rad Laboratories, Richmond, CA). Electroporated cells were immediately diluted in RPMI 1640 supplemented with 10% FCS, washed once, resuspended in complete medium, and plated in 24-well culture plates. 1% DMSO was added immediately after transfection and after 16–24 h the cells were treated with 100 U/ml of murine recombinant IFN-γ (gift from Dr. G. Garotta, Human Genome Sciences Inc., Rockville, MD) in the presence or absence of 100 nM $1,25(OH)_2D_3$ (Hoffmann-La Roche Inc.). 8 h later the cells were stimulated with 0.1% *Staphylococcus aureus* Cowan I strain (SAC) (Calbiochem Corp., La Jolla, CA) or by the addition of J558L cells expressing CD40L. After an additional 16 h the cells were harvested

to assay luciferase and β-galactosidase activities. The specific plasmid DNA was complemented with an irrelevant plasmid DNA to reach 150 μg of total DNA per transfection. 40 μg of each p40 promoter/PXP2 construct was used in each transfection. 20 μg of VDR, RXRα, and empty expression vectors were used as indicated in the figure legends. 10 μg of CMV-β-galactosidase expression plasmids was used for normalization of transfection efficiency of luciferase reporter constructs.

Luciferase and β-galactosidase assays. Transfected cells were washed twice with PBS and lysed with ice cold lysis buffer containing 1% Triton-X 100, 25 mM glycylglycine, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, pH 8, 2 mM ATP, and 1 mM dithiothreitol. Luciferase assay was performed in a buffer containing 25 mM glycylglycine, 15 mM $MgSO_4$, and 5 mM ATP. 250 μl of assay buffer and 50 μl of clarified cell lysate were combined before reading in a standard luminometer (model LB 9407; EG and G Berthold, Berthold, Germany) using automated injection of 100 μl of a 1 mM solution of synthetic cristalline D-luciferine (Sigma Chemical Co.) in 25 mM glycylglycine. The β-galactosidase assay was performed in a buffer containing 7.4 mM sodium phosphate buffer, pH 7.3, 1 mM $MgSO_4$, 10 mM KCl, 50 mM β-mercaptoethanol, and 0.8 mg/ml *O*-nitrophenyl-β-D-galactopyranoside. 20 μl of clarified cell lysate was incubated in a 200-μl buffer vol for 30 min and then inactivated by adding 100 μl of 1 M Na_2CO_3. Readings were taken at OD_{405} in a standard spectrophotometer.

Nuclear extracts and gel shift assays. THP-1 cells (2.5×10^8) were cultured for 24 h in complete medium containing 1% DMSO and subsequently in the presence or absence of 100 U/ml of IFN-γ for an additional 16 h. The cells were incubated in the presence or absence of 500 nM $1,25(OH)_2D_3$ for 2 h and subsequently treated with 0.1% SAC for an additional 2 h before harvesting. Nuclear extracts were obtained following the procedure described by Dignam et al. (51). Nuclear proteins were quantitated by the Bradford method (Bio-Rad Laboratories) and stored at −80°C. For gel shift analysis the double-stranded oligonucleotides were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase according to standard protocols. 5–10 μg of nuclear extracts from THP-1 cells was incubated with 0.1–0.5 ng of labeled probes ($20–30 \times 10^3$ cpm) for 20 min at room temperature in 20 μl buffer containing 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 0.3 mg/ml BSA, and 2 μg of poly (dI-dC). In the experiment shown in Fig. 7 *B* an additional ~ 50 ng of double-stranded oligonucleotide of unrelated sequence was included in the reaction to further reduce nonspecific binding. Unlabeled oligonucleotide competitors (5 ng) were added before the addition of nuclear extracts. For the inhibition assays 1 μg of antibodies to p65 or p50 was added to the nuclear extracts 10 min before adding the probe. The reactions were analyzed by electrophoresis in a nondenaturing 5% polyacrylamide gel in 0.5× TBE. The gels were then dried and exposed at −80°C for autoradiography. The radioactivity in specific protein–DNA complexes was quantified by exposure to a PhosphorImager (model GS-525; Bio-Rad Laboratories).

Results

$1,25(OH)_2D_3$ inhibits IL-12 production by myelomonocytic cells. We examined the effect of $1,25(OH)_2D_3$ on the production of IL-12 by freshly isolated human monocytes primed with IFN-γ and stimulated with SAC (Fig. 1 *A*). $1,25(OH)_2D_3$ inhibits the production of IL-12 in a dose-dependent manner with a 50% inhibitory concentration (IC_{50}) of 1 nM. $1,25(OH)_2D_3$ does not influence IL-10 production by SAC-stimu-

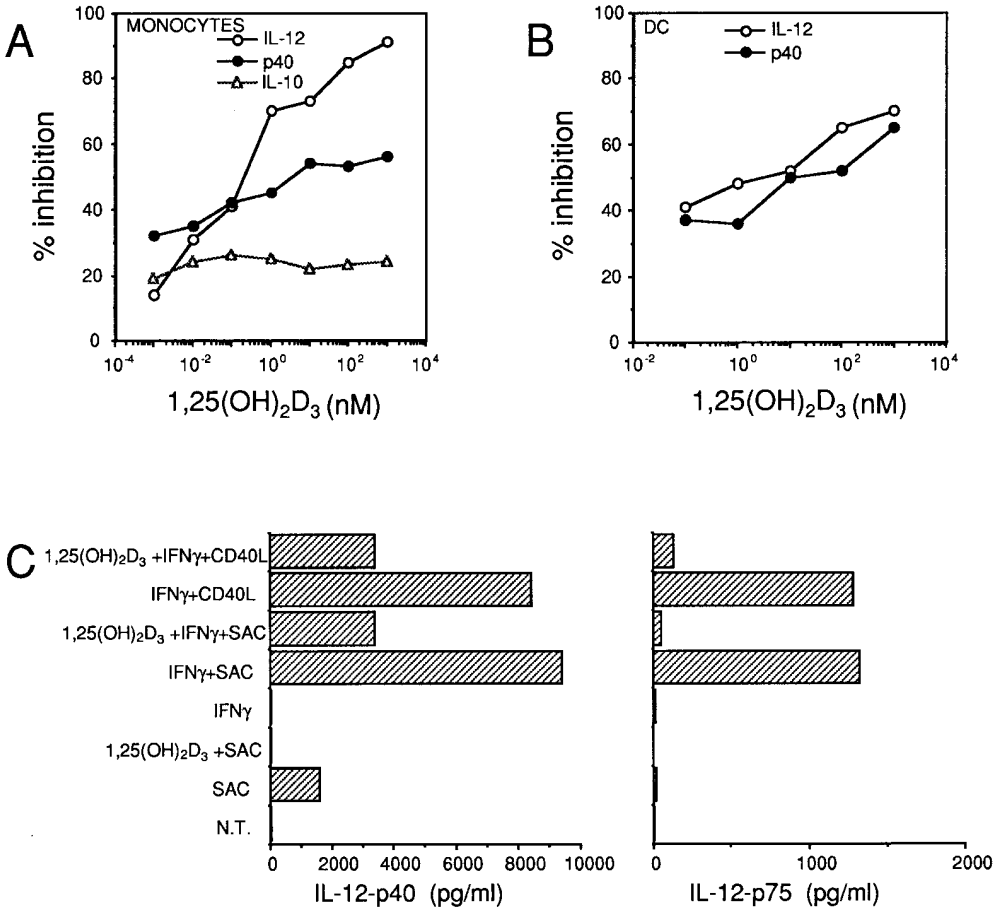

Figure 1. $1,25(OH)_2D_3$ specifically inhibits IL-12 production by human monocytes and DCs. (*A*) Human monocytes were primed with IFN-γ (1,000 U/ml) for 16 h and stimulated with SAC (0.1%) in the presence of different concentrations of $1,25(OH)_2D_3$. Cytokine concentrations were evaluated by ELISA. Cytokines levels in the absence of $1,25(OH)_2D_3$ were as follows: IL-12, 800 pg/ml; p40, 1.8 ng/ml; IL-10, 1.4 ng/ml. (*B*) Human DCs were isolated as described in Methods and stimulated for 24 h with IFN-γ (1,000 U/ml) and J558-mCD40L transfectants in the presence of different concentrations of $1,25(OH)_2D_3$. Cytokine levels in the absence of $1,25(OH)_2D_3$ were as follows: IL-12, 2.4 ng/ml; p40, 4 ng/ml. (*C*) THP-1 cells were primed with IFN-γ (1,000 U/ml) for 16 h and stimulated either with SAC (0.1%) (*left*) or J558-mCD40L transfectants (*right*) for 24 h in the presence or absence of $1,25(OH)_2D_3$. Results are representative of three different experiments.

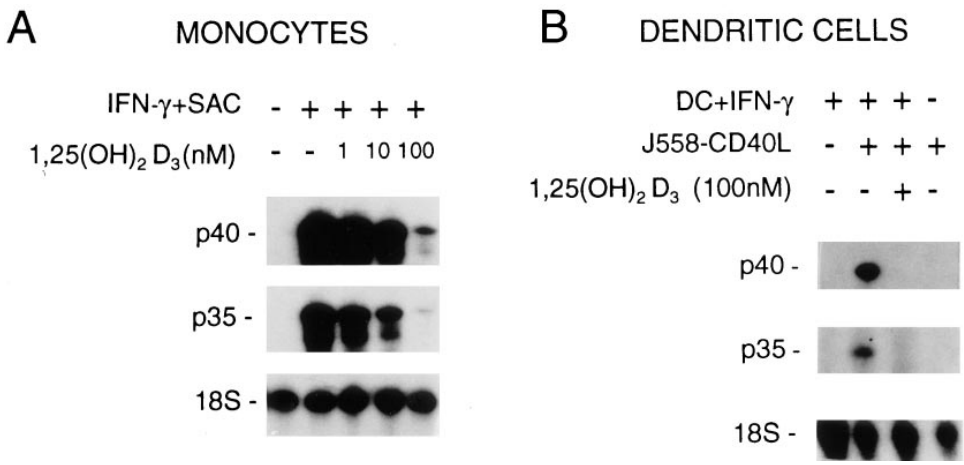

Figure 2. (*A*) $1,25(OH)_2D_3$ inhibits expression of IL-12 p40 and p35 mRNA in human monocytes. The inhibition of IL-12 p40 and p35 mRNA expression by $1,25(OH)_2D_3$ was analyzed in human monocytes primed with IFN-γ for 16 and then stimulated with SAC for 4 h in the presence or absence of different concentrations of $1,25(OH)_2D_3$. (*B*) The inhibition of IL-12 p40 and p35 mRNA expression was analyzed in DC stimulated with IFN-γ (1,000 U/ml) and J558-mCD40L cells in the presence or absence of $1,25(OH)_2D_3$. RNA was extracted and IL-12 p40 and p35 transcripts were analyzed as indicated in Methods. Protected fragments were precipitated and fractionated by polyacrylamide/urea 1× TBE gel. The 18S message was used as a control. A representative experiment is shown out of three similar experiments performed.

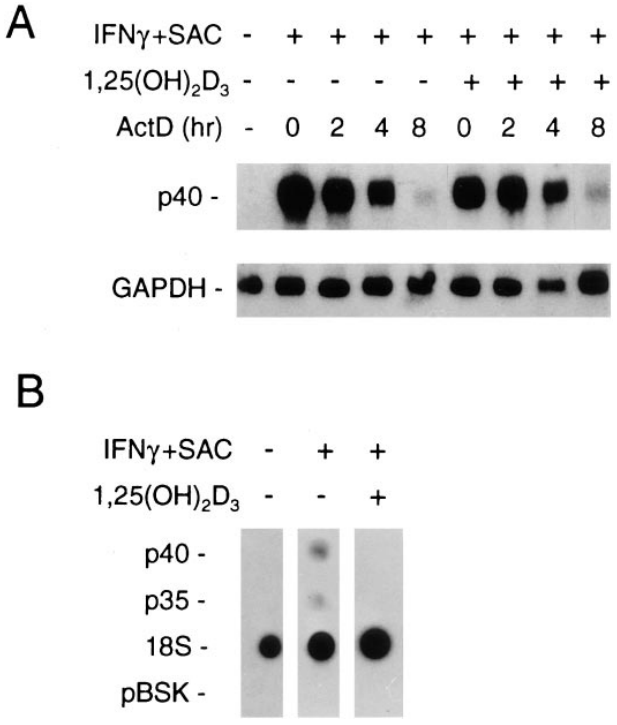

Figure 3. $1,25(OH)_2D_3$ does not accelerate IL-12 p40 mRNA degradation and inhibits IL-12 p40 and p35 gene transcription in human monocytes. (*A*) Human monocytes were primed with IFN-γ for 16 h and then stimulated with SAC in the presence or absence of $1,25(OH)_2D_3$ (10 nM). Actinomycin D (10 μg/ml) was added 4 h after SAC stimulation. RNA was isolated by Trizol at 0, 2, 4, and 8 h after addition of actinomycin D, and subject to Northern blot analysis. Gels were dried and exposed to PhosphorImager screens and the p40 and p35 messages were normalized against the glyceraldehyde-3-phosphate dehydrogenase message. (*B*) Transcriptional rate was analyzed by nuclear run-on assays performed on isolated nuclei prepared from human THP-1 cells primed with IFN-γ for 16 h and then stimulated with SAC for 4 h in the presence or absence of $1,25(OH)_2D_3$. [α-^{32}P]UTP-labeled nuclear RNA was hybridized for 84 h to p40, p35, and 18S cDNA (1 μg/slot) immobilized on nylon membranes. The membranes were dried, exposed and subject to PhosphorImager analysis. A representative experiment is shown out of four similar experiments performed.

lated monocytes (Fig. 1 *A*). Similar results were obtained using whole peripheral blood mononuclear cells instead of purified monocytes (data not shown).

Since DC represent an important source of IL-12 (52, 53), we asked whether $1,25(OH)_2D_3$ could also inhibit IL-12 production by these cells. DC can be triggered to produce very high levels of IL-12 upon ligation of CD40 by CD40L (10, 11). Therefore, we stimulated human monocyte-derived DC with IFN-γ and J558 cells transfected with the mouse CD40 ligand in the presence or absence of $1,25(OH)_2D_3$ (Fig. 1 *B*). Production of both IL-12 p75 and p40 is inhibited in a dose-dependent manner. To eliminate the possibility that the inhibition could be the result of an indirect action on contaminating cells present in the monocyte and DC preparations, we tested the effects of $1,25(OH)_2D_3$ on the human monocytic cell line THP-1. Inhibition of SAC-mediated as well as CD40-triggered IL-12 p75 and p40 secretion was also observed in THP-1 cells (Fig. 1 *C*), confirming a direct effect on monocytes.

$1,25(OH)_2D_3$ inhibits IL-12 p40 and p35 mRNA expression and transcription rate. To determine whether the inhibition of IL-12 secretion is the result of decreased mRNA production, we analyzed the effect of $1,25(OH)_2D_3$ on the expression of IL-12 p40 and p35 mRNA in SAC-stimulated monocytes. At a concentration of 10 nM, $1,25(OH)_2D_3$ induces ~ 40% inhibition of p40 and p35 mRNA, as compared with the untreated control (Fig. 2 *A*). Total RNA was isolated from four DC preparations stimulated with IFN-γ and J558-mCD40L cells in the presence or absence of $1,25(OH)_2D_3$, and expression of IL-12 p40, p35, and 18S mRNAs was evaluated by RNase protection assay (Fig. 2 *B*). A strong inhibition of both p40 and p35 mRNA accumulation was observed in the presence of $1,25(OH)_2D_3$. The effect of $1,25(OH)_2D_3$ on IL-12 p40 mRNA stability was examined by blocking transcription with actino-

mycin D after stimulation with SAC. As shown in Fig. 3 *A*, $1,25(OH)_2D_3$ does not accelerate IL-12 p40 mRNA degradation. The $t_{1/2}$ of SAC-induced p40 mRNA is 4 h and it is not altered by addition of $1,25(OH)_2D_3$.

To directly test whether inhibition of p40 and p35 mRNA occurs at the level of transcriptional initiation, nuclear run-on experiments were performed on isolated nuclei prepared from PBMC primed with IFN-γ and stimulated with SAC. $1,25-(OH)_2D_3$ inhibits transcription of both genes (Fig. 3 *B*). Similar levels of inhibition of p40 and p35 mRNA expression and gene transcription were observed. This suggests that most of the inhibition of IL-12 subunits expression occurs at the transcriptional level.

Transcriptional repression of the p40 gene by $1,25(OH)_2D_3$ requires coexpression of VDR and RXR and a functional VDR DNA binding domain. Having shown that $1,25(OH)_2D_3$ represses transcriptional activation of p35 and p40 IL-12 subunits, and given that p40 is the highly inducible and tightly regulated component of IL-12 (7), we further investigated the molecular basis of transcriptional repression of the p40 gene. To explore the effect of $1,25(OH)_2D_3$ on transcriptional regulation of the p40 gene, we transiently transfected the murine monocytic cell line RAW264.7 with p40 promoter/reporter constructs. We were unable to use THP-1 cells for this purpose because of low transfection efficiency. Therefore, we used RAW264.7 cells because of their amenability to transfection and their capacity to produce p40 in response to IFN-γ and SAC (12). In addition, proper regulation of human p40 promoter in these cells has been documented previously (12). The −292/+56 p40 promoter/reporter construct was transfected in RAW264.7 cells together with expression vectors for VDR, RXRα, or both and cells were treated subsequently with $1,25(OH)_2D_3$ or diluent and then stimulated or left untreated. The analysis of luciferase activity in cell extracts from trans-

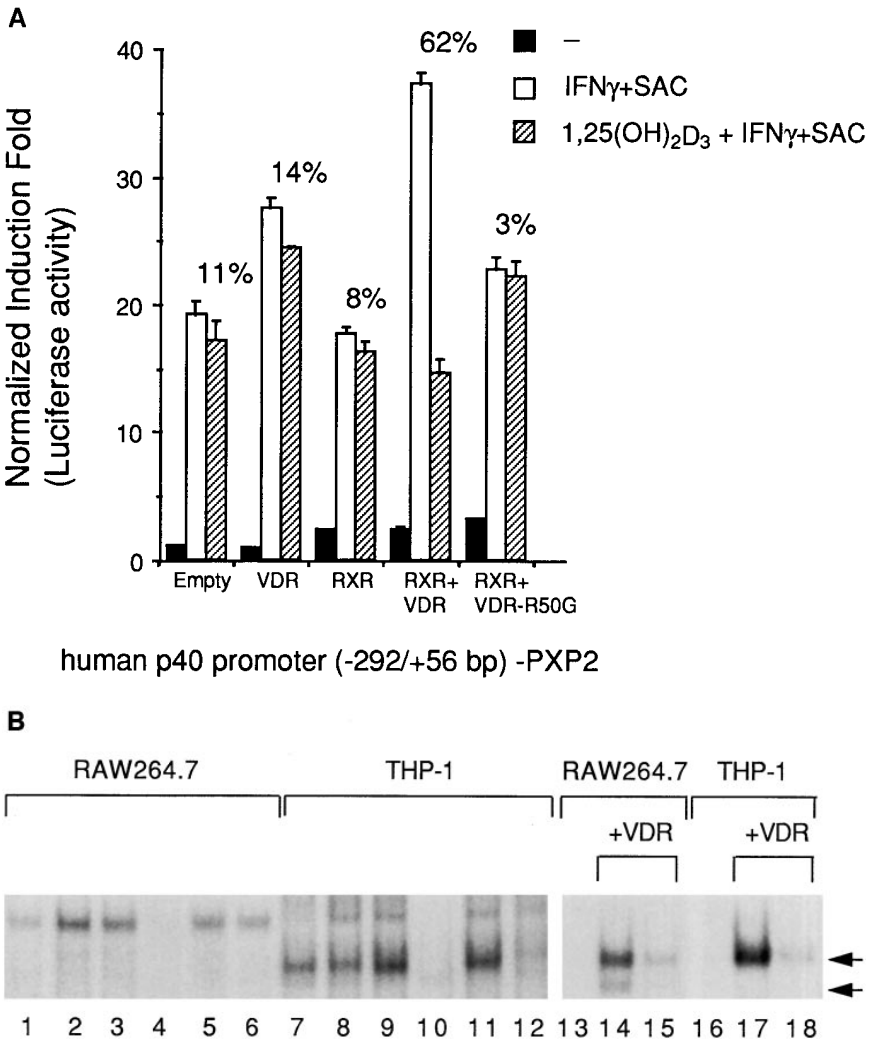

Figure 4. (*A*) Transcriptional suppression of IL-12 p40 gene by $1,25(OH)_2D_3$ requires coexpression of VDR and RXR. Transient transfection of RAW264.7 with the human p40 promoter (−292/+56 bp)-luciferase reporter PXP2 construct. Cells were cotransfected with expression vectors for RXR and/or VDR or a VDR DNA–binding domain mutant (R50G) in which R-50 was mutated to G. 1% DMSO was added immediately after transfection, 16 h later the cells were stimulated with IFN-γ with or without $1,25(OH)_2D_3$ for 8 h and subsequently stimulated with SAC for 16 h. Luciferase activity was normalized against an internal cotransfected β-galactosidase standard. The results are expressed as induction fold over the value obtained in the untreated condition (−) of the sample cotransfected with empty expression vector, which was given an arbitrary value of 1. The percentages of inhibition induced by treatment with $1,25(OH)_2D_3$ are indicated above the bars. The experiment shown was performed in duplicate and is representative of four similar experiments. (*B*) VDRE binding complexes in RAW264.7 and THP-1 cells. 10 μg (lanes *1–12*) or 5 μg (lanes *13–18*) of nuclear extracts from THP-1 (lanes *7–12* and *16–18*) or RAW264.7 (lanes *1–6* and *13–15*) cells was incubated with [^{32}P]-labeled oligonucleotide probe with the sequence: 5′-agctTCGCGGGTGAACGGGGGCA-GAGCA-3′ from the VDRE of the human osteocalcin gene. Extracts were prepared from cells either left untreated (lanes *1*, *4*, *7*, *10*, and *13–18*), stimulated with 0.1% SAC for 2 h (lanes *2*, *5*, and *8*) or treated with 100 nM $1,25(OH)_2D_3$ for 2 h and then stimulated with 0.1% SAC for 2 h (lanes *3–6* and *9–12*). 100 ng of recombinant VDR protein was added to assess the presence of RXRs in the nuclear extracts (lanes *14*, *15*, *17*, and *18*). Unlabeled VDRE oligonucleotide was added to compete specific complexes (lanes *4* and *10*). Anti-VDR (lanes *6*, *12*, *15*, and *18*) or control (lanes *5*, *11*, *14*, and *17*) antibodies were added to identify VDR-containing complexes. Specific protein–DNA complexes containing VDR were resolved by EMSA and are indicated by arrows. Heterodimeric VDR–RXR complexes migrate more slowly than homodimeric VDR–VDR complexes. RAW264.7 cells exhibit barely detectable VDRE binding complexes whereas THP-1 cells show the presence of abundant VDRE binding complexes. Addition of recombinant VDR to THP-1 cell extract results in strong binding of VDR–RXR complexes, whereas in RAW264.7 cells VDR–RXR complexes are present at a significantly lower level and an additional faster migrating complex is formed by VDR, indicating that RXR is limiting in RAW264.7.

fected cells confirms inducibility of the −292/+56 bp p40 promoter region in response to stimulation with IFN-γ and SAC (Fig. 4) or CD40L expressing J558L cells (data not shown). Treatment with $1,25(OH)_2D_3$ results in an ∼ 10% decrease in activation (Fig. 4 *A*), which correlates with a weak inhibition of endogenous p40 production possibly due to the very low expression of hormone receptor in these cells when compared with THP-1 cells (Fig. 4 *B*). Overexpression of VDR or RXRα has a modest effect on inhibition, whereas simultaneous overexpression of VDR and RXRα results in 60% inhibition of p40 promoter activity (Fig. 4 *A*). These data confirm that $1,25(OH)_2D_3$ negatively regulates transcriptional activation of the p40 gene and further indicate that the inhibition may depend on binding of $1,25(OH)_2D_3$ to VDR and subsequent formation of VDR-RXR heterodimers. To verify the requirement for VDR DNA binding activity in the $1,25(OH)_2D_3$-mediated inhibition of p40, we coexpressed the −292/+56 p40 promoter construct with a DNA binding domain mutant of VDR, which fails to bind to VDRE consensus sequences (43). The analysis of luciferase activity expressed in transfected cells reveals that the mutant tested is unable to inhibit p40 transcriptional activity, indicating that an intact VDR DNA–binding domain is required for the inhibition (Fig. 4 *A*).

Part of the inhibitory effect of $1,25(OH)_2D_3$ maps to the NF-κB binding site in the p40 promoter. Since $1,25(OH)_2D_3$ induces VDR-dependent inhibition of p40 transcriptional activation, we wished to identify the region involved in negative regulation of the p40 gene. Based on previously published work which identified important regulatory sequences at positions −222/−204 and −117/−101 bp relative to the transcription start site (12–14), we generated p40 promoter/reporter constructs scanning these regions. The constructs encompassed p40 promoter sequences starting at positions −292, −250, −224, −180 and ending at position +56 bp relative to the transcription initiation site (Fig. 5 *A*). RAW264.7 cells were transfected with these p40 promoter/reporter constructs together with expression vectors for VDR and RXRα. Since CD40-mediated activation of p40 promoter has not been reported, and $1,25(OH)_2D_3$ is able to block IL-12 production induced by CD40 triggering (Figs. 1 and 2), we compared activation by SAC with that provided by J558L-CD40L expressing cells. Consistent with the study of Ma et al. (12), deletion of sequences down to position −224 bp does not diminish promoter activation, while further deletion to position −180 bp significantly decreases activation in response to stimulation with SAC (Fig. 5 *B*, *left*) or CD40L expressing cells (Fig. 5 *B*, *right*). Importantly, the inhibitory effect of $1,25(OH)_2D_3$ is fully retained within −180 bp from the transcription initiation site (Fig. 5 *B*) resulting in 60–70% inhibition regardless of the mode of activation. These findings suggest that the target of $1,25(OH)_2D_3$ action may reside within this −180-bp region. In addition, our data show that CD40-triggering regulates expression of the p40 gene very similarly to SAC-mediated stimulation. It is noteworthy that the −180/+56 bp region contains a previously characterized NF-κB binding site mapping at position −117/−101 bp, which has been implicated in p40 promoter activation (14). Since the −180/+56 bp region lacks any VDRE consensus sequence, we hypothesized that $1,25(OH)_2D_3$ may inhibit expression of the p40 gene by interfering with the function of the p40-κB site. To test the role of the p40-κB site in $1,25(OH)_2D_3$-mediated inhibition of p40 gene transcription, we generated a −292/+56 bp p40 promoter/

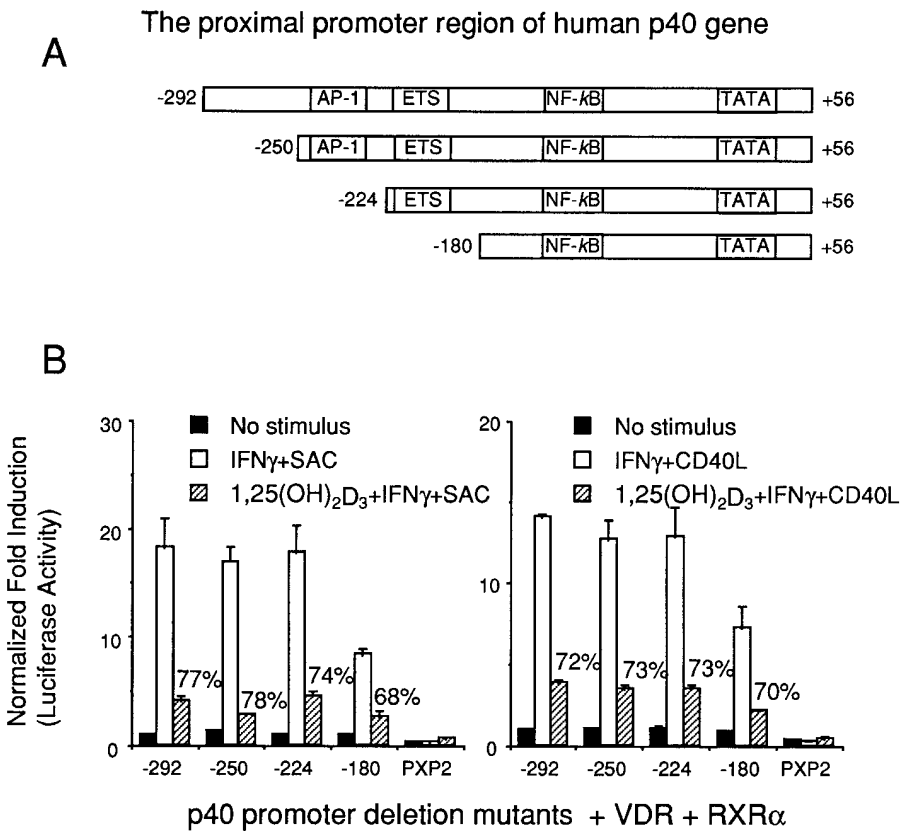

Figure 5. Analysis of $1,25(OH)_2D_3$-mediated transcriptional repression of p40 promoter 5′ deletion constructs activated by SAC or CD40 triggering. (*A*) Schematic representation of the human p40 promoter sequence (−292/+56 bp) indicating putative nuclear factor binding sites, and 5′ deletion mutants of p40 promoter. (*B* and *C*) Transient transfection of RAW264.7 cells with 5′ deletion mutants of the p40 promoter/luciferase reporter PXP2 construct. Stimulation was performed with SAC (*B*) or J558L CD40L expressing cells (*C*). The results are expressed as induction fold over the value obtained in the untreated condition (no stimulus) of the sample transfected with the −292/+56-p40 reporter construct, which was given an arbitrary value of 1. The percentages of inhibition induced by treatment with $1,25(OH)_2D_3$ are indicated above the bars. The experiment shown was performed in duplicate and is representative of three similar experiments.

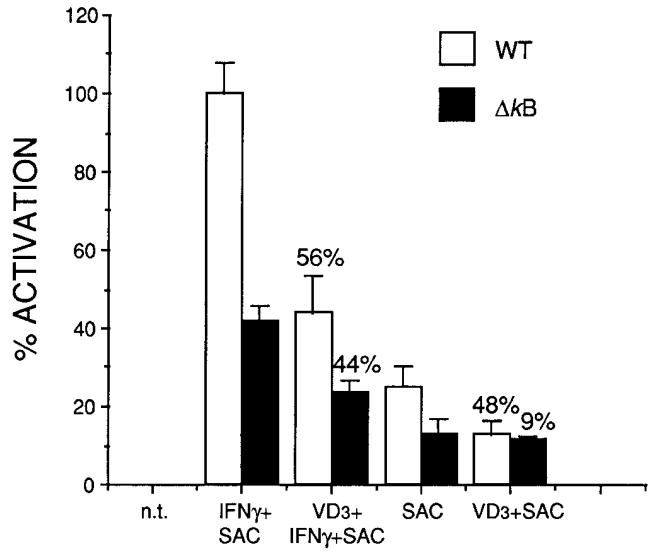

Figure 6. Involvement of the p40-κB site in $1,25(OH)_2D_3$-mediated transcriptional repression of the p40 gene. Transient transfection of RAW264.7 cells with wild-type or a p40-κB internal deletion mutant (ΔκB) 292/+56 bp p40 promoter/luciferase reporter constructs. Transfected cells were treated with $1,25(OH)_2D_3$ or left untreated and then stimulated with SAC only or IFN-γ and SAC. The normalized induction fold of the wild-type p40 promoter stimulated with IFN-γ and SAC was taken as 100% activation and other data points were related to this value. The percentages of inhibition after treatment with $1,25(OH)_2D_3$ (indicated as *VD_3*) were calculated as in Fig. 4 and are indicated above the bars. The experiment shown is representative of four similar experiments performed.

reporter construct bearing an internal deletion of this site (ΔκB). The inhibitory effect of the hormone on activation of wild-type and ΔκB p40 promoter was analyzed in RAW264.7 cells transfected with VDR and RXR expression vectors. Consistent with recent findings (12), our experiments show that priming with IFN-γ results in an approximately fourfold increase in p40 promoter activation (Fig. 6). Furthermore, deletion of the p40-κB site results in ~ 50% reduction of promoter activation confirming the importance of this site in p40 gene expression. Nevertheless, activation of the ΔκB construct was measurable under these experimental conditions and permitted analysis of the effect of $1,25(OH)_2D_3$. Addition of $1,25(OH)_2D_3$ to cells primed with IFN-γ and stimulated with SAC results in 56% inhibition of wild-type and 44% inhibition of ΔκB promoter activities (Fig. 6). Addition of the hormone to unprimed SAC-stimulated cells results in 48 and 9% inhibition of wild-type and ΔκB promoter activities, respectively (Fig. 6). Together these observations indicate that although the p40-κB site is involved, additional regions also play a role in negative regulation of p40 promoter activity by $1,25(OH)_2D_3$.

NF-κB binding to the p40-κB sequence is partially inhibited by $1,25(OH)_2D_3$. To gain more insight into the mechanisms of $1,25(OH)_2D_3$-mediated inhibition of p40-κB function, we analyzed the binding activity present in nuclear extracts of THP-1 cells, to a double-stranded oligonucleotide with the sequence of the p40-κB site. Stimulated THP-1 cells exhibit p40-κB binding activity. This activity is specific since: it is absent in nonstimulated cells, it is inhibited by antibodies to p65 and p50 NF-κB components, and it is competed with by a consensus Ig-κB sequence but not by a mutant sequence (Fig. 7 *A*). To assay the effect of $1,25(OH)_2D_3$ on NF-κB activation, THP-1 cells were incubated in the presence or absence of IFN-γ, treated with $1,25(OH)_2D_3$, then stimulated with SAC and subsequently harvested to prepare nuclear extracts. The analysis of p40-κB binding activity from these extracts reveals that preincubation with $1,25(OH)_2D_3$ leads to a decrease in p40-κB binding activity (Fig. 7 *B*, *top*). In contrast, binding to an octamer site is constitutive and is not affected by $1,25(OH)_2D_3$ (Fig. 7 *B*, *bottom*). When NF-κB binding activity was quantified and normalized against octamer binding activity, the inhibition of p40-κB binding activity was calculated to range from 35 to 50% (Fig. 7 *C*).

Based on previous work showing the direct inhibition of NFAT/AP-1 complex by VDR (43), it was conceivable that VDR-RXR complexes could interfere with NF-κB binding to the p40-κB sequence. To address this possibility, we incubated the p40-κB sequence with recombinant purified VDR and/or RXR in the presence or absence of nuclear extracts from stimulated THP-1 cells. This experiment reveals no direct interaction of VDR-VDR homodimers or VDR-RXR heterodimers with the p40-κB sequence and no inhibition of endogenous NF-κB binding (data not shown). Thus, the partial inhibition of NF-κB binding is likely to be the result of an indirect action of the hormone–receptor complex on NF-κB activation or function.

Discussion

It has been reported recently that $1,25(OH)_2D_3$ inhibits IL-12 production by activated macrophages (25). In this report we confirm and provide a molecular basis for those findings. We demonstrate that $1,25(OH)_2D_3$ inhibits the secretion of the IL-12 heterodimer, as well as its p40 subunit. The inhibitory effect is observed on monocytes as well as on DC. The lack of inhibition of IL-10 secretion indicates that the effect on IL-12 is not the result of a general dampening of cellular activation. Moreover, the action of $1,25(OH)_2D_3$ does not depend on the type of stimulus provided, being equally effective in inhibiting SAC-induced or CD40-mediated IL-12 production. $1,25(OH)_2D_3$ has also been shown to inhibit secretion of IL-2 and IFN-γ by T lymphocytes (33, 34, 54). Given that IFN-γ production provides a positive feedback loop for IL-12 secretion by macrophages (2, 7), the action on both cytokines may amplify the negative effects of $1,25(OH)_2D_3$ on Th1 development in vivo.

Expression of p35 and p40 subunits is upregulated in response to stimuli which induce IL-12 secretion. Although both subunits are regulated primarily at the transcriptional level, their mode of regulation may differ (13, 15, 16). Analysis of the effect of $1,25(OH)_2D_3$ on the expression of IL-12 subunits reveals that the hormone inhibits expression of both p35 and p40 mRNA in monocytes as well as in DC. Our data also show that $1,25(OH)_2D_3$ inhibits the expression of p40 and p35 at the transcriptional level. These findings may explain the potent inhibitory effect of the hormone on IL-12 p75 secretion. It is possible that by decreasing the expression of both IL-12 subunits, $1,25(OH)_2D_3$ may prevent reaching the threshold required for the assembly and secretion of the heterodimer.

Although expression of both subunits is necessary for IL-12

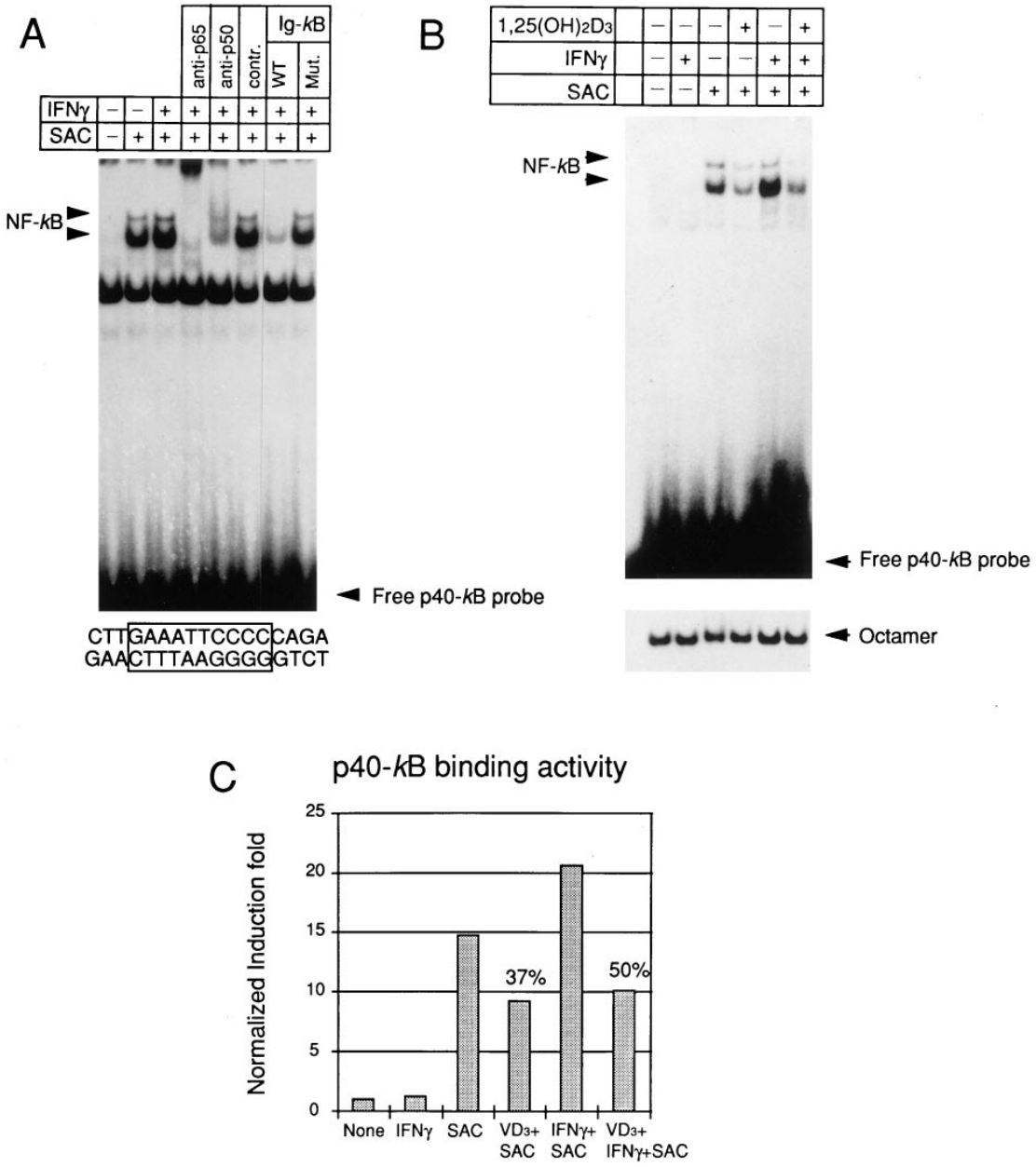

Figure 7. $1,25(OH)_2D_3$ decreases NF-κB binding to the p40-κB sequence. (*A*) Inducible binding of NF-κB to the p40-κB sequence in nuclear extracts of THP-1 cells. Cells were primed with IFN-γ for 16 h (lanes *3–8*), stimulated subsequently with SAC for 4 h (lanes *2–8*), and then harvested to make nuclear extracts. Nuclear extracts were incubated with anti-p65 (lane *4*), anti-p50 (lane *5*), or control (lane *6*) rabbit polyclonal antibodies. The binding to the labeled p40-κB sequence was competed by incubation with a 20-fold excess of unlabeled oligonucleotide with a consensus (lane *7*) or a mutant (lane *8*) Ig-κB binding site sequence. (*B*) p40-κB (*above*) and octamer (*below*) binding in nuclear extracts of THP-1 cells left untreated (lane *2*) or stimulated with IFN-γ (lane *3*), SAC (lanes *4* and *5*), IFN-γ and SAC (lanes *6* and *7*). $1,25(OH)_2D_3$ was added 2 h before stimulation with SAC (lanes *5* and *7*). Migration of specific NF-κB-DNA complexes and of free ^{32}P-labeled double-stranded oligonucleotide with the p40-κB sequence are indicated by arrowheads. The faster migrating complex present in *A* is nonspecific. (*C*) Normalized NF-κB binding activity from the experiment in *B*). The signals of NF-κB–specific complexes were quantified by PhosphorImager and normalized against the signals of octamer-specific complexes. The results are expressed as induction fold over the value obtained in the untreated sample (lane *2* of *B*). The percentages of inhibition induced by treatment with $1,25(OH)_2D_3$ are indicated above the bars.

production by myelomonocytic cells, p35 inducibility appears to be modest, whereas expression of p40 is highly inducible and primarily regulated at the transcriptional level (12). Previous work identified a region, extending 292 bp upstream to the transcription start site, responsible for most of the transcriptional regulation of the p40 gene (12). The effect of $1,25(OH)_2D_3$ was assessed by transient transfection of p40 promoter/reporter constructs in RAW264.7 cells. We observed a modest inhibition of p40 secretion and transcriptional activity in this cell line likely due to low expression of hormone receptor. The low expression level of hormone receptor in RAW264.7 may reflect a more mature phenotype of these cells

as VDR expression levels have been reported to decline in maturing macrophages (31). Simultaneous overexpression of VDR and RXRα greatly enhances the $1,25(OH)_2D_3$-mediated inhibition. VDR has been reported to bind to cognate DNA sequences in the form of VDR-VDR homodimers or VDR-RXR heterodimers and to consequently regulate gene transcription (55, 56). The different composition of the receptor complex influences its ability to bind to and transactivate from certain VDREs (41). Moreover, a mutation in the VDR DNA–binding domain that abolishes its ability to recognize specific DNA sequences abrogates the capacity of the hormone to inhibit p40 gene transcription. Together these findings indicate that the inhibition of p40 expression by $1,25(OH)_2D_3$ is a VDR-mediated event possibly involving formation of VDR-RXR heterodimers and binding to specific DNA sequences.

Analysis of the p40 promoter does not reveal the presence of any putative VDR binding sites. To map the site of action of $1,25(OH)_2D_3$, we analyzed the inhibitory effect of the hormone on a series of 5′ deletions of the p40 promoter. The inhibition is retained within 180 bp upstream of the transcription initiation site, suggesting that $1,25(OH)_2D_3$ may interfere with the inducible binding of NF-κB at position −117/−101 bp in the p40 promoter. Deletion of the p40-κB site indicates that this site plays a role in transcriptional repression of the p40 gene, although it is not the only target of $1,25(OH)_2D_3$. The relative contribution of the p40-κB site was significantly greater when SAC stimulation was performed in the absence of IFN-γ, suggesting that additional regulatory elements induced by the synergistic action of IFN-γ and SAC may be targets for the action of $1,25(OH)_2D_3$. Recent reports established a role for IRF-1 in regulation of IL-12 production and Th1 cell development (57, 58). In addition, inducible binding of IRF-1 to the p40 promoter has been documented (13). IRF-1 is known to participate in the regulation of IFN-γ–inducible genes such as MHC class I (59). However, IRF-1 is unlikely to be a target of $1,25(OH)_2D_3$ given that the hormone inhibits IFN-γ–inducible class II, but has no effect on class I expression on fresh monocytes (60) or THP-1 cells (D'Ambrosio, D., unpublished observation). Clearly more work will be required to identify additional targets of inhibition by $1,25(OH)_2D_3$. Nevertheless, involvement of the p40-κB site is supported by a recent report demonstrating downregulation of NF-κB activation in T lymphocytes treated with $1,25(OH)_2D_3$ (44). The study mentioned above (44) also demonstrated that $1,25(OH)_2D_3$ inhibits transcriptional activation of a multimerized κB reporter, indicating that the hormone inhibits NF-κB–driven transcription. Our data confirm those findings by showing that NF-κB binding to the p40-κB sequence is significantly decreased by $1,25(OH)_2D_3$. We have attempted to assess the effect of $1,25(OH)_2D_3$ on a trimerized p40-κB reporter. Unfortunately, although some inhibition was seen, the results of this experiment were difficult to interpret due to nonspecific effects of the hormone on the control vector and overall low inducibility of this construct in our experimental conditions. Despite the fact that our transfection data indicate the requirement of VDR and RXR for optimal inhibition of p40 gene transcription, we have failed to detect direct binding of purified VDR and/or RXR proteins to the p40-κB site or displacement of the NF-κB complex. This suggests an indirect effect of the hormone–receptor complex on the p40-κB site. In their study, Yu et al. have shown that $1,25(OH)_2D_3$ inhibits the activation-dependent upregulation of NF-κB components (44). Since expression of NF-κB components is subject to autoregulation by NF-κB itself (61), the inhibition could result from an early block in activation of NF-κB as well as from inhibition of p50 and c-Rel gene expression. It is noteworthy that we observe decreased binding of NF-κB to the p40-κB site in response to $1,25(OH)_2D_3$, even 1 h after activation (data not shown). Together these findings support the hypothesis that $1,25(OH)_2D_3$ interferes with the molecular events leading to activation and/or nuclear translocation of NF-κB. Moreover, it is important to note that a multiprotein complex containing c-Rel and binding to the p40 promoter in response to IFN-γ and SAC has been reported recently (13). These observations suggest that by downregulating NF-κB, $1,25(OH)_2D_3$ may also affect the function of this multiprotein complex and possibly explain the high residual inhibition that we observe in the ΔκB p40 promoter stimulated with IFN-γ and SAC. These findings also suggest that the hormone may affect the expression of multiple genes which are subject to regulation by NF-κB. A similar situation is seen in the case of glucocorticoids, which have been shown to interfere with the initial activation and nuclear translocation of NF-κB complexes by upregulating the expression of the inhibitor IκBα (62, 63). Indeed, glucocorticoids also inhibit IL-12 production by monocytes (Panina-Bordignon, P., unpublished observations). However, $1,25(OH)_2D_3$ does not significantly upregulate the expression of IκBα. Further work will be required to elucidate the mechanism(s) by which $1,25(OH)_2D_3$ inhibits NF-κB. It is noteworthy that the published sequence of the p35 promoter also contains a consensus binding site for NF-κB (15, 16). Even though the functional relevance of this site awaits confirmation, it is tempting to speculate that by downregulating NF-κB activity, $1,25(OH)_2D_3$ could inhibit the expression of both IL-12 subunits.

Activated monocytes are able to produce and respond to $1,25(OH)_2D_3$ (31, 64, 65), suggesting that the hormone may provide an autocrine negative regulatory loop to downregulate the production of proinflammatory cytokines such as IL-12. Although the beneficial effects of $1,25(OH)_2D_3$ in treatment of animal models of autoimmunity has been documented recently (19–23), the hypercalcemia caused by the hormone has limited its therapeutic applications. The development of novel $1,25(OH)_2D_3$ analogues with more efficient immunosuppressive properties and reduced hypercalcemic effects may provide new therapeutic tools for the treatment of chronic inflammatory autoimmune disorders.

Acknowledgments

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